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Epidermal Sensing of Oxygen Is Essential for Systemic Hypoxic Response

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SUMMARY

Skin plays an essential role, mediated in part by its remarkable vascular plasticity, in adaptation to environmental stimuli. Certain vertebrates, such as amphibians, respond to hypoxia in part through the skin; but it is unknown whether this tissue can influence mammalian systemic adaptation to low oxygen levels. We have found that epidermal deletion of the hypoxia-responsive transcription factor *HIF-1α* inhibits renal erythropoietin (EPO) synthesis in response to hypoxia. Conversely, mice with an epidermal deletion of the von Hippel-Lindau (VHL) factor, a negative regulator of HIF, have increased EPO synthesis and polycythemia. We show that nitric oxide release induced by the HIF pathway acts on cutaneous vascular flow to increase systemic erythropoietin expression. These results demonstrate that in mice the skin is a critical mediator of systemic responses to environmental oxygen.

INTRODUCTION

Mammalian skin acts as an essential buffer against the environment (Tobin, 2006). In this role, the skin can act to protect internal tissues as a barrier, e.g., by conserving water and guarding against pathogens. It can also respond to environmental stresses. These latter changes are accomplished in part by regulated alterations in blood flow through the cutaneous circulation.

Cutaneous vascular flow in mammals controls a wide range of physiological parameters through an intricate system of vascular plexi (Tobin, 2006). Body heat is tightly linked to both the external environment and internal metabolic processes, and the relationship of skin and surface area to metabolism is one of the oldest concerns of biology. For example, some of the first formulas relating oxygen use, skin/surface area, metabolism, and heat were proposed in the mid-1800s, in the pioneering work of Bergmann and Rubner (Bergmann, 1847; Rubner, 1883).

Among vertebrates, systemic metabolism is closely tied to dermal physiology, particularly in amphibians where the skin has a clear and important respiratory function. In mammals this relationship has been relatively unexplored. However, one recent study has argued that human epidermis does not obtain its oxygen from the dermal circulation but rather utilizes oxygen directly from the atmosphere (Stucker et al., 2002). Thus, skin may be unique as a tissue in not being directly reliant on cardiopulmonary delivery of oxygen for its survival. Interestingly, when the air overlying the epidermis becomes hypoxic, keratinocytes are able to induce vasodilation in the underlying dermal vasculature, in a nitric oxide-dependent fashion (Minson, 2003). This vasodilation is independent of changes in respiration or temperature and may allow oxygen delivery to the keratinocytes under circumstances where insufficient oxygen is present in the atmosphere.

A primary mammalian response to hypoxia is the increased synthesis of the hormone erythropoietin (EPO), an erythropoietic agent that is chiefly produced by the kidney and liver (Fandrey, 2004). Several nonerythropoietic roles for EPO have been demonstrated, and it has also been shown that a number of tissues and cells outside of the hematopoietic system express the EPO receptor (EPO-R) (Lewis, 2004). These include neurons as well as vascular endothelial cells and cardiac myocytes, all of which are susceptible to damage during hypoxic insult (Gassmann et al., 2003).

Recent work has shown that EPO and its receptors are key factors in ventilatory adaptations to hypoxia (Soliz et al., 2007). Thus the induction of EPO synthesis may be one of the most primary responses of the body to lowered oxygen levels, influencing or even coordinating a cascade of systemic responses to hypoxia that extend beyond erythropoiesis.

Differential vascular flow in the kidney can influence EPO expression, which is coupled to changes in renal blood gas levels acting on oxygen-sensing cells near the proximal tubules of the renal nephron (Fandrey, 2004). The molecular mechanisms for this involve hypoxia-inducible transcription factors (HIFs), which are primary modulators of the transcriptional hypoxic response (Semenza, 2004) and are negatively regulated by the von Hippel-Lindau (VHL) factor (Ivan et al., 2001; Maxwell et al., 2001). The *VHL* gene acts to ubiquitinate the *HIF-α* transcription factors (*HIF-1α* and *HIF-2α*) and induce their turnover in normoxia due to recognition of a prolyl hydroxylation motif, wherein oxygen-dependent hydroxylases modify the HIF-α proteins.

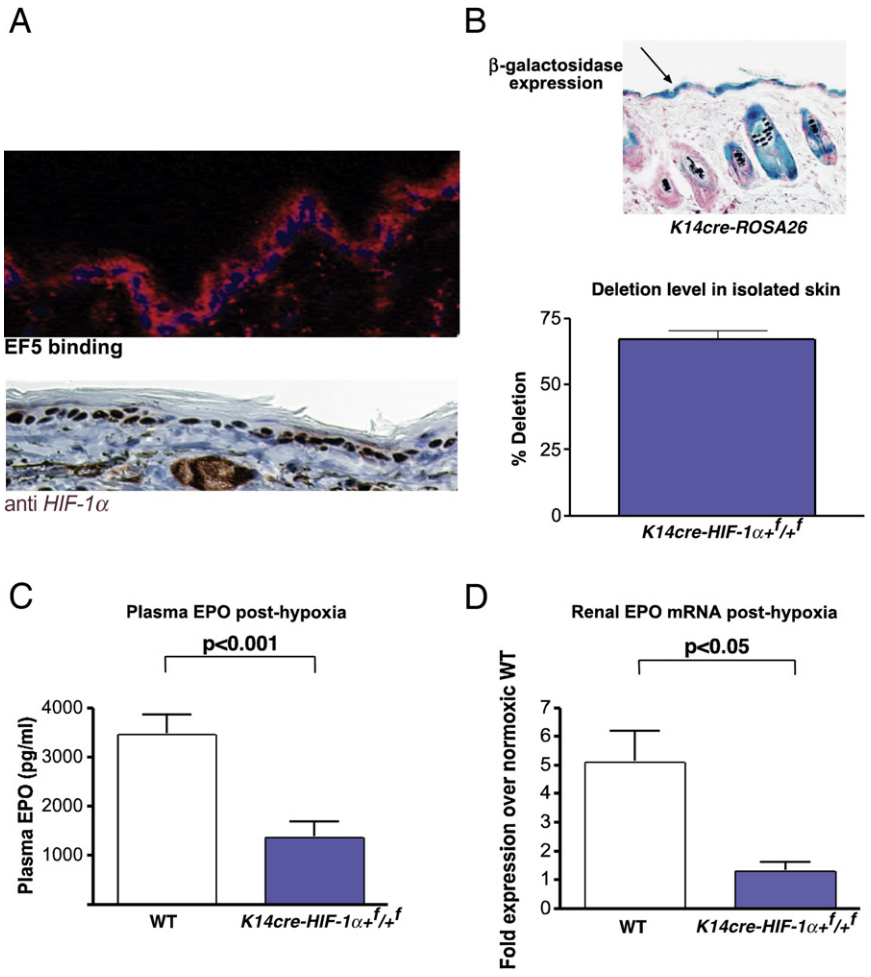

Figure 1. Loss of *HIF-1α* in the Epidermis Diminishes Renal EPO Production during Hypoxia

(A) The normal mouse epidermis is hypoxic, based on EF5 binding (red), and expresses *HIF-1α* protein (red).

(B) K14 cre recombinase transgene expression was verified by β-galactosidase expression in epidermis in the ROSA25 reporter strain; deletion efficiency of the loxP-flanked allele in the epidermis and hair follicle was 70%.

(C) EPO protein in the plasma following hypoxic exposure for 14 hr at 9% oxygen is significantly reduced in mice lacking *HIF-1α* in the epidermis (wild-type (WT), n = 25; *K14cre-HIF-1α*$^{+f/+f}$, n = 11).

(D) Renal EPO mRNA expression is reduced to normoxic levels in hypoxic *K14cre-HIF-1α*$^{+f/+f}$ mice (WT, n = 6; *K14cre-HIF-1α*$^{+f/+f}$, n = 3). All graphs represent mean ± standard error of the mean (SEM).

Loss of *VHL* prevents the ubiquitination of hydroxylated HIF-α proteins and causes increases in HIF-mediated transcription due to accumulation of the transcription factors (Ivan et al., 2001; Maxwell et al., 1999, 2001). Increased HIF-α levels in the skin can contribute to vascular expansion and thus alteration of cutaneous vascular flow (Elson et al., 2001; Kim et al., 2006b). We wished to determine whether this altered HIF-α function can influence physiological homeostasis, and, more generally, whether the skin plays a role in hypoxic adaptive responses in mammals.

We provide evidence that the skin is a primary coordinator of the systemic hypoxic response and acts to modulate cutaneous blood flow to potentiate renal and hepatic EPO synthesis. This occurs in a clearly HIF-dependent manner. These findings indicate a previously unappreciated and fundamental role for mammalian skin in responding to environmental oxygenation.

RESULTS

HIF-1α Is Extensively Expressed in the Normal Epidermis

It has been previously shown that normal rodent and human skin has many characteristics of a constitutively hypoxic tissue, including the binding of hypoxia-detection agents, e.g., the nitroimidazole EF5 (Evans et al., 2006). Mouse epidermis demonstrates extensive binding of this hypoxia-sensitive compound, particularly in basal keratinocytes (Figure 1A). Consistent with a constitutive low level of tissue oxygenation are high levels of expression of the hypoxia-inducible transcription factor *HIF-1α* in the nuclei of keratinocytes (Figures 1A and S3 available online).

Loss of *HIF-1α* in the Epidermis

To determine the role of *HIF-1α* expression in the epidermis, *HIF-1α* was deleted tissue specifically by crossing the *HIF-1α*$^{+f/+f}$ allele with a strain expressing cre recombinase driven by the keratin-14 (K14) promoter (Jonkers et al., 2001). This transgene limits expression to basal keratinocytes and a small number of other epithelial lineages (Jonkers et al., 2001). We verified tissue specificity of cre recombinase expression by a cross of this transgene into the ROSA26 cre reporter strain (Soriano, 1999), which gave rise to cre-induced β-galactosidase expression in epidermis (Figure 1B). No β-galactosidase expression was seen in brain, liver, kidney, lung, or other visceral organs (data not shown), indicating the *K14cre* transgene is not active in those tissues. Deletion analysis of genomic DNA by real-time PCR also indicated extensive deletion in the epidermis of *K14cre-HIF-1α*$^{+f/+f}$ mice, and no deletion was detectable in liver or kidney (Figure 1B and data not shown).

Loss of *HIF-1α* in Keratinocytes Prevents a Systemic Hypoxic Response

K14cre-HIF-1α$^{+f/+f}$ mutant animals develop normally (data not shown). Given the central role for EPO in the physiological response to hypoxia, including its modulation of adaptive mechanisms such as erythropoiesis and ventilation (Soliz et al., 2007), we focused on it as a key readout of systemic responses to hypoxia.

Basal expression of EPO was unchanged in *K14cre-HIF-1α*$^{+f/+f}$ mutant animals, and their hematocrits were also normal (data not shown). To test hypoxic response, we placed *K14cre-HIF-1α*$^{+f/+f}$ mice in chambers in which they were subjected to normobaric hypoxia (9% O_2) for 14 hr. This level of hypoxia induces an approximately 30-fold increase in plasma levels of EPO in wild-type (WT) mice, an increase from basal levels of approximately 100 pg/ml plasma to a mean of approximately 3500 pg/ml (Figure 1C). We found that blood EPO concentration in the mutants following this hypoxic challenge were only 30% of those found in WT animals (Figure 1C). This was correlated with a loss of hypoxically induced EPO expression in the kidney: renal EPO mRNA expression was not significantly elevated in mutants (Figure 1D).

This demonstrates that a *HIF-1α*-dependent response in the skin is an important element for triggering renal synthesis of EPO. We carried out similar experiments on *K14cre-HIF-2α*$^{+f/+f}$ mutants; however, we saw no difference in hypoxia-induced plasma EPO levels in these animals compared to WT littermates (data not shown). This indicates that epidermal induction of EPO is a *HIF-1α*-mediated response, differentiating it from the role played by *HIF-2α* in the direct regulation of EPO in the kidney (Gruber et al., 2007).

Deletion of *VHL* in the Epidermis

To further study the mechanisms underlying HIF response in the epidermis, a model for constitutively increased HIF expression was employed. This utilized a tissue-specific deletion of a negative regulator of HIF-α function, the *VHL* gene (Maxwell et al., 1999). This deletion results in upregulation of both *HIF-1α* and *HIF-2α* in keratinocytes (Haase, 2005; Kim et al., 2006a).

Loss of *VHL* in the epidermis causes an approximate 20% increase in vascularization of the skin (Kim et al., 2006b). As shown in Figure 2A, it also causes increases in HIF target gene expression.

Constitutive or Induced Loss of *VHL* in the Epidermis Dramatically Increases Blood EPO Levels

Adult *K14cre-VHL*$^{+f/+f}$ mutant animals have high levels of plasma EPO (Figure 2B). This is accompanied by an increase in reticulocytes (Figure S1A) and a significant increase in hematocrit, to an approximate level of 0.70 (Figure 2D). Skin barrier function is normal, and there is no evidence of dehydration (data not shown), indicating that the high hematocrit is due to a high level of erythropoiesis. Blood volume is significantly higher than that found in WT animals and is consistent with a nonleaky vasculature and an expansion of blood volume due to an increase in erythrocyte mass (Figure S1A).

Induced Deletion of *VHL* also Induces EPO Expression

Since the *K14cre* transgene is expressed as early as embryonic day 14.5 (Vasioukhin et al., 1999), it was important to ascertain whether this dramatic increase in EPO production was related to the role for HIF described above, and thus represented a physiological stimulation of the EPO pathway via the skin, or whether it is due to some developmental alteration in EPO regulation.

To separate physiological from developmental effects, we crossed the *VHL*$^{+f/+f}$ allele into a conditional K14cre-transgenic background. This conditional transgenic strain allows tamoxifen treatment to be used to induce keratinocyte-specific deletion of a loxP-flanked allele in adults (Vasioukhin et al., 1999). As seen in Figure 2B, 6 weeks after tamoxifen-induced cre recombinase activation in 4-week-old mice, plasma EPO levels had risen significantly compared to WT control littermates treated with tamoxifen. This demonstrates that loss of *VHL* in the skin induces systemic EPO elevation even when the epidermis has developed normally.

Extensive analysis of protein and mRNA found no evidence that either skin or isolated keratinocytes are capable of expressing EPO under normoxia or hypoxia, nor when *VHL* deletion occurs (data not shown). All EPO expression is thus generated in these mutant mice through physiological signaling.

Hepatic EPO Expression in Constitutive *VHL* Mutants Is Correlated with High Hematocrit

We found that in the constitutively deleted *K14cre-VHL*$^{+f/+f}$ mutant animals, the liver has the highest levels of increased EPO expression (Figure 2C). Generally, the kidney, rather than the liver, is the predominant site of basal and induced EPO synthesis. To determine whether this shift in site of EPO expression was specific to epidermal signaling, tissue EPO expression levels in *K14TAMcre-VHL*$^{+f/+f}$ mice were analyzed 6 weeks after induced deletion. As can be seen in Figure 2C, mean expression levels are increased in the kidney, and not the liver, of these mice.

Next we analyzed EPO levels, synthesis, and hematocrit in WT and *K14cre-VHL*$^{+f/+f}$ 10-day-old pups and in mature animals at 10 weeks (Figure 2D). In the pups, hematocrits are not different, although *K14cre-VHL*$^{+f/+f}$ mutant plasma EPO levels are significantly elevated. Both hepatic and renal EPO mRNA synthesis are slightly elevated in 10-day-old pups. However, at 10 weeks of age, renal EPO mRNA expression has been suppressed compared to WT, and only hepatic EPO mRNA levels are still high. This is correlated with a high hematocrit in the adult *K14cre-VHL*$^{+f/+f}$ mutant. Suppression of renal EPO mRNA synthesis by a high hematocrit has been documented by others in studies of hypobaric hypoxia (Bozzini et al., 2005; Lezon et al., 1995); since young *K14cre-VHL*$^{+f/+f}$ mice and induced deletion *K14TAMcre-VHL*$^{+f/+f}$ mice both have elevated renal EPO mRNA expression, the epidermal *VHL* mutation can affect both the kidney and the liver physiologically.

Double and Triple Deletions of HIF-αs and *VHL* in Skin Demonstrate a Predominant Role for *HIF-2α*

To address the mechanisms responsible for EPO response induced by *VHL* deletion in the skin, a genetic analysis was carried out to determine whether the effects from *VHL* deletion are directly HIF related since the *VHL* gene product has been proposed to regulate non-HIF targets (Russell and Ohh, 2007). Double and triple deletions were carried out by crossing *HIF-1α* and *HIF-2α* conditional alleles alone, and in combination, into the background of the *VHL* conditional and *K14cre* alleles (Figure 3A). Deletion of *HIF-1α* lowers mean serum EPO levels, but deletion of *HIF-2α* causes a dramatic reduction, restoring them to WT levels. Interestingly, deletion of both *HIF-1α* and *HIF-2α*

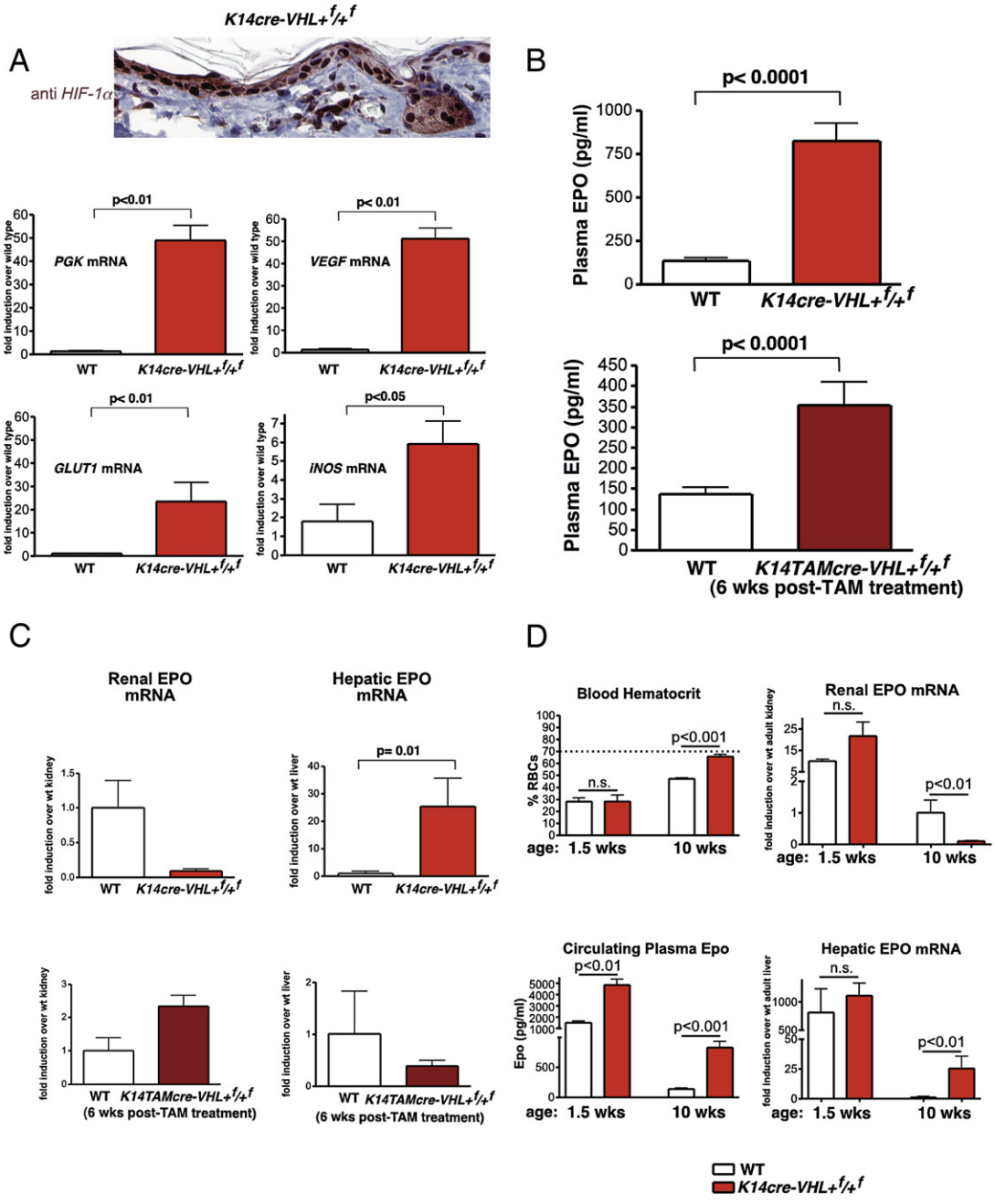

Figure 2. Deletion of *VHL* in the Epidermis Induces EPO Production during Normoxia

(A) Upon deletion of *VHL* in the epidermis, *HIF-1α* protein (red) is stabilized and HIF target gene expression is increased in the skin (for each graph, WT, n = 3; *K14cre-VHL*$^{+f/+f}$, n = 3 for RNA isolation).

(B) Constitutive or Tamoxifen-induced epidermal deletion of *VHL* results in highly elevated plasma EPO (WT, n = 36; *K14cre-VHL*$^{+f/+f}$, n = 23, *K14TAMcre-VHL*$^{+f/+f}$, n = 5).

(C) In the *K14cre-VHL*$^{+f/+f}$ mouse, EPO mRNA expression is suppressed in the kidney but increased in the liver. In the tamoxifen-inducible *K14TAMcre-VHL*$^{+f/+f}$ mice, where deletion occurs in the adult, EPO expression is increased in the kidney and unaffected in the liver (WT, n = 9; *K14cre-VHL*$^{+f/+f}$, n = 6; *K14TAMcre-VHL*$^{+f/+f}$, n = 5).

(D) As blood hematocrit levels increase in the constitutively deleted *K14cre-VHL*$^{+f/+f}$ mice (WT 1.5 weeks, n = 3; *K14cre-VHL*$^{+f/+f}$ 1.5 weeks, n = 4; WT 10 weeks, n = 43; *K14cre-VHL*$^{+f/+f}$ 10 weeks, n = 32), renal EPO mRNA expression is suppressed and hepatic EPO increases, indicating that hematocrit can selectively affect renal EPO expression (WT 1.5 weeks, n = 4; *K14cre-VHL*$^{+f/+f}$ 1.5 weeks, n = 5; WT 10 weeks, n = 9; *K14cre-VHL*$^{+f/+f}$ 10 weeks, n = 6). All graphs represent mean ± SEM.

causes a decrease in EPO to levels significantly below those seen in WT mice (Figure 3A).

In Figure 3B it can be seen that loss of *HIF-2α*, but not *HIF-1α*, restores hematocrit in *K14cre-VHL*$^{+f/+f}$ mice to WT levels. This indicates that the drop in EPO seen in Figure 3A, following loss of *HIF-1α*, was not sufficient to affect erythropoiesis. These data together indicate that *VHL* deletion is acting to effect changes in EPO expression through *HIF-2α*. As discussed above, the epidermal deletion of *HIF-1α* (and not *HIF-2α*) alone affects systemic hypoxic response; but as shown here *VHL* deletion causes *HIF-2α* to act as the primary transcription factor in the same response. Thus this is evidence that gene regulation via the HIF pathway can differentially employ *HIF-1α* or *HIF-2α*, dependent on *VHL* status. This coincides with experimental observations in other settings of *VHL* loss of function that indicate that loss of *VHL* preferentially increases *HIF-2α* activity (Carroll and Ashcroft, 2006; Kim et al., 2006b; Kondo et al., 2002; Rankin et al., 2007; Raval et al., 2005; Scortegagna et al., 2005).

NO Levels and Blood Flow Shifts Correlate with Increasing EPO Levels

To address physiological mechanisms underlying this HIF-mediated effect on systemic erythropoiesis, we assayed blood oxygen and blood pressure levels in *K14cre-VHL*$^{+f/+f}$ mutants (Figure 3C). Here we saw no significant effects on overall blood oxygenation but a highly significant decrease in blood pressure in *K14cre-VHL*$^{+f/+f}$ mutants (Figure 3C). Since blood flow through the renal and hepatic circulatory beds is a key determinant of organ oxygenation and hypoxic response, we wished to determine whether differential changes in blood flow could be occurring in *K14cre-VHL*$^{+f/+f}$ mutant mice. To assay this, we injected fluorescent microspheres into the left atria of experimental mice; these microspheres lodge in capillaries and their distribution relative to tissue mass within an animal gives a ratio of differential blood flow to differing tissues. As can be seen in Figure 3D, there is a significant shift in flow toward the skin, and away from the liver and kidney, in *K14cre-VHL*$^{+f/+f}$ mutant mice.

To assay whether this shift in blood flow was accompanied by a change in tissue oxygenation, we injected animals with the nitroimidazole EF5 to determine whether there was an increase in the binding of this hypoxia marker in *K14cre-VHL*$^{+f/+f}$ mutant kidney and liver, and thus a change in tissue oxygenation (Figure 3E). Interestingly, while there was a trend to increased hypoxia in the kidney, it was only significantly increased in the liver of *K14cre-VHL*$^{+f/+f}$ mutant mice (Figure 3E). This agrees with data (Figure 2C) demonstrating that the liver has a 25-fold increased EPO expression in the *K14cre-VHL*$^{+f/+f}$ mutant animals.

Role of NO in Mediating Cutaneous Induction of EPO Synthesis

Nitric oxide (NO) is a critical mediator of cutaneous vasodilation in response to local heat, injury, and hypoxia (Harbrecht, 2006; Houghton et al., 2006) and can induce vascular hypotension. One key target gene of the HIF transcriptional response in many tissues is the inducible nitric oxide synthase gene (*iNOS*) (Figure 2A). As can be seen in Figure 3F, *K14cre-VHL*$^{+f/+f}$ mice have a highly significant increase in plasma NO, indicating that a large increase in NO synthesis has occurred in mutants relative to WT animals.

We next wished to determine the role played by increased NO levels in the increased synthesis of EPO and, in particular, establish whether an NO-induced shift in dermal circulation could act to reduce blood flow to tissues such as the liver and kidney. We first established whether alterations in systemic, as opposed to tissue-specific, NO would increase EPO synthesis. This is important in part because significant evidence indicates that NO is capable of inducing HIF activation/stabilization (Hagen et al., 2003; Mateo et al., 2003; Metzen et al., 2003). It has been shown in rats that broad pharmacological inhibition of NO synthesis causes systemic increases in EPO production (Todorov et al., 2000). We found the same to be true of mice (Figure 4A): after 4 days of treatment with the NO synthesis inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME), plasma EPO levels rise approximately 20% in WT mice. Thus systemic inhibition of NO synthesis acts to raise EPO production. A simple explanation for this observation would be a role for NO-mediated vasorelaxation in directly increasing blood flow to the kidney and liver, absence of NO thus decreasing flow and increasing EPO response.

Inhibition of NO Synthesis Specifically Blocks Dermal Induction of EPO Synthesis

To determine more specifically the role of the HIF target gene *iNOS* in EPO regulation, we assayed the hypoxic response of mice with a global deletion of the NOS2 gene encoding *iNOS* (Laubach et al., 1995). These mice showed no differences in basal levels of plasma EPO or in hematocrit relative to WT mice (data not shown); but they did show a highly significant increase in EPO response following 14 hr of hypoxia (Figure 4A). This demonstrates again that reduced systemic NO can increase EPO signaling. The global nature of the *iNOS* knockout does prevent drawing conclusions about tissue-specific effects of NO production, however.

To assay for the role of NO production via epidermal deletion of *VHL*, we treated the *K14cre-VHL*$^{+f/+f}$ mice with the NO synthase inhibitor L-NAME and found that 4 days of treatment significantly lowered EPO levels in mutant mice, reducing them to levels seen in WT mice treated with this compound (Figure 4B). This is the opposite of the effect of L-NAME seen in either the WT mice described above or the *iNOS*$^{-/-}$ mice and indicates that NO signaling is essential in the special case of HIF-mediated upregulation of EPO in the skin. This in turn argues for a direct link between *VHL* deletion in the skin, NO synthesis, and changes in physiology leading to EPO expression.

Dermal, but Not Systemic, Administration of NO Donor Increases EPO Levels

The observation above suggests an intriguing method to induce EPO expression. We either treated C57Bl6 mice with a systemic nitric oxide donor (nitroglycerin) via oral gavage of a slow release formulation or used a similar dosing of nitroglycerin through a patch on the skin. As is shown in Figure 4C, systemic administration of the NO donor does not result in an increase in EPO after 7 hr of treatment; however, epidermal administration causes an almost 7-fold rise in plasma EPO levels. Plasma NO metabolite levels at 7 hr are similar following both treatments (Figure S2A);

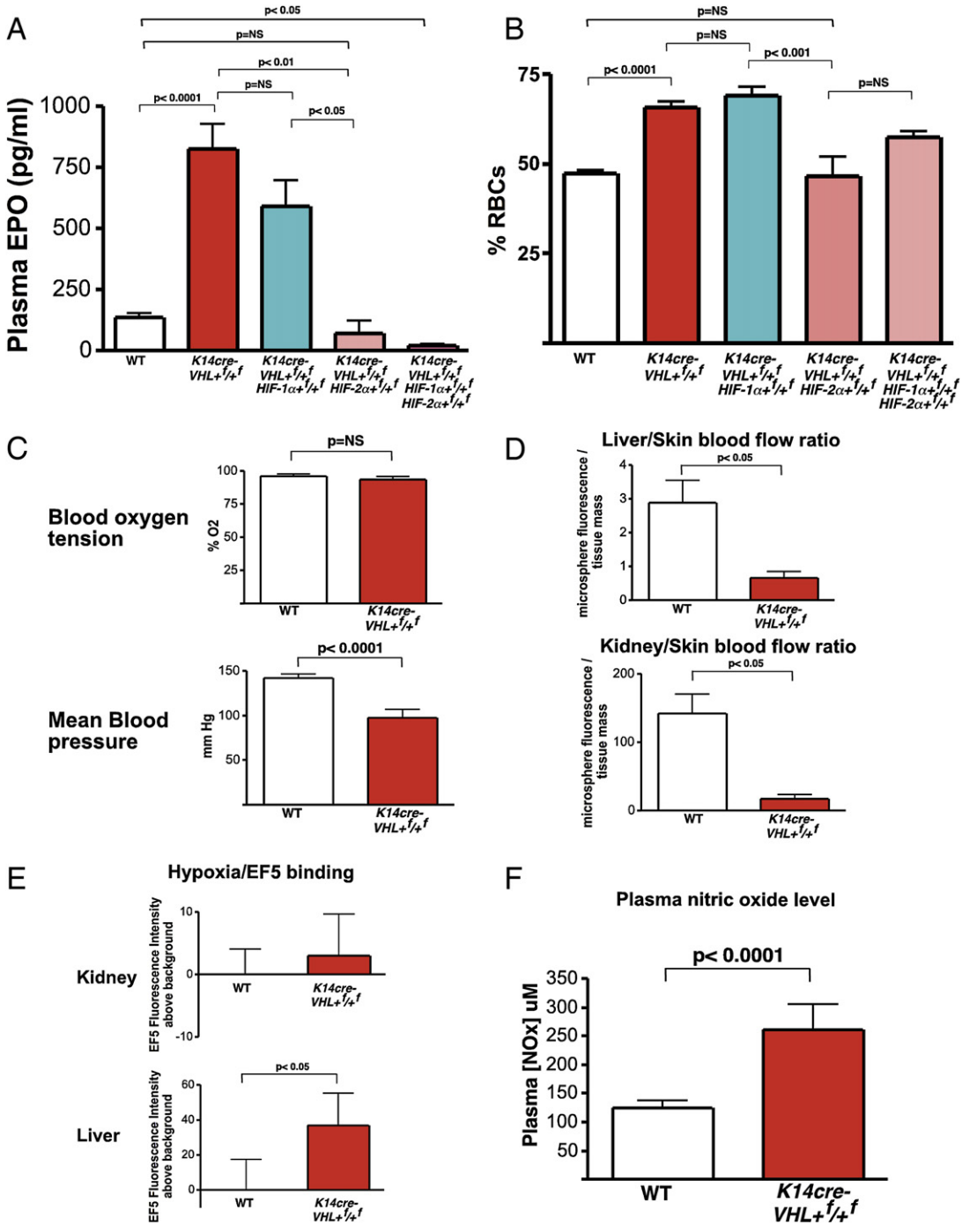

Figure 3. *K14cre-VHL*$^{+f/+f}$ Demonstrates Altered Blood Flow, Increased Internal Hypoxia, Increased EPO Expression, and Restoration to WT Levels by Co-deletion of *HIF-2α*

(A) Deletion of *HIF-2α* but not *HIF-1α* in the *K14cre-VHL*$^{+f/+f}$ background restores plasma EPO (WT, n = 36; *K14cre-VHL*$^{+f/+f}$, n = 23; *K14cre-VHL*$^{+f/+f}$ *HIF-1α*$^{+f/+f}$, n = 15; *K14cre-VHL*$^{+f/+f}$ *HIF-2α*$^{+f/+f}$, n = 3; *K14cre-VHL*$^{+f/+f}$ *HIF-1α*$^{+f/+f}$ *HIF-2α*$^{+f/+f}$, n = 3) and blood hematocrit (B) to WT levels (WT, n = 43; *K14cre-VHL*$^{+f/+f}$, n = 32; *K14cre-VHL*$^{+f/+f}$ *HIF-1α*$^{+f/+f}$, n = 17; *K14cre-VHL*$^{+f/+f}$ *HIF-2α*$^{+f/+f}$, n = 3; *K14cre-VHL*$^{+f/+f}$ *HIF-1α*$^{+f/+f}$ *HIF-2α*$^{+f/+f}$, n = 3). Deletion of both *HIF-1α* and *HIF-2α* is similar in effect to deletion of *HIF-2α* alone.

(C) Blood oxygen saturation is normal in *K14cre-VHL*$^{+f/+f}$ animals (WT, n = 7; *K14cre-VHL*$^{+f/+f}$, n = 6), but animals are hypotensive (WT, n = 17; *K14cre-VHL*$^{+f/+f}$, n = 10).

(D) Blood flow in *K14cre-VHL*$^{+f/+f}$ mice is shifted away from the liver and kidney and toward the skin, as measured by microsphere distribution (WT, n = 11; *K14cre-VHL*$^{+f/+f}$, n = 4).

however, only epidermal administration causes a significant rise in renal EPO synthesis (Figure 4D). It was subsequently found that other stimuli of epidermal NO release, such as mustard oil, also induce significant renal EPO synthesis when administered cutaneously, and that concurrent L-NAME treatment partially blocks this induction (Figure S1B).

As can be seen in Figures 4E and 4F, epidermal administration of nitroglycerin shifts blood flow away from the splanchnic sites of EPO production and significantly induces hypoxia as visualized by increased EF5 binding in those organs after treatment.

Short-Term Hypoxic Response Reveals a Differential Role for the Skin in EPO Response to Hypoxia

The data above demonstrate an important role for HIF response in the skin in the regulation of EPO synthesis. However, a great deal of adaptation to hypoxia occurs immediately upon exposure to low oxygen through changes in heart and respiration rates and through pulmonary vasoconstriction (Powell et al., 1998). As part of this physiological response, EPO synthesis begins almost immediately (Abbrecht and Littell, 1972).

In acute hypoxia, blood is distributed toward the brain and liver and is shunted away from the skin (Kuwahira et al., 1993). Acute hypoxia also induces an immediate, ion-channel-mediated vasoconstriction in the lung (Moudgil et al., 2005, 2006); interestingly, this same phenomenon has been shown to occur within seconds in the skin of amphibians (Malvin and Walker, 2001).

We wished to determine whether acute responses to hypoxia are influenced by changes in skin oxygenation. To test this, we placed C57Bl6 WT mice in chambers that enclosed their heads in one compartment and their bodies in a separately ventilated compartment (Figures 5A and S1C). We then determined the level of EPO response following respiration and skin exposure to different levels of normobaric oxygen for 5 hr. As can be seen in Figure 5B, there is no effect from changing skin exposure when mice are breathing normally oxygenated air. As expected, a large increase in EPO levels is seen when mice respire in a hypoxic environment.

Surprisingly, when mice have their bodies exposed to normoxia while they breath hypoxic air, the hypoxic EPO response is more than doubled relative to animals that are both breathing and have body exposure to hypoxia. It is clear that this exposure affects renal EPO synthesis since there is a correlated doubling of EPO mRNA in the kidney in these mice (Figure 5C).

We wished to determine whether these changes were also correlated with a shift in vascular flow from the skin toward splanchnic organs. Repeating the experiment above, with a shorter (1 hr) time of exposure, it was found that in this acute response, normoxia surrounding the body while mice are breathing hypoxic air caused a shift in blood flow toward the skin and significantly reduced relative flow to the liver (Figure 5D). The mean shift was an almost 10-fold change relative to mice both breathing and surrounded by a hypoxic environment. This finding demonstrates that there is an acute hypoxic response in the skin that modulates vascular flow and regulates systemic hypoxic response. Interestingly, there is evidence that this acute response may be mediated by the same oxygen-sensitive potassium channels that control pulmonary vasoconstriction. We found that Kv1.5 potassium channels, which are essential for hypoxia-induced pulmonary vasoconstriction (Moudgil et al., 2006), are also present in cutaneous blood vessels in the skin (Figure S1D).

To determine the relationship of these findings to *HIF-1α* function, we carried out the experiments described above on *K14cre-HIF-1α*$^{+f/+f}$ mutant animals. This revealed that *HIF-1α* also plays a key role in the regulation of the immediate adaptation to hypoxia. As shown in Figure S2C, *K14cre-HIF-1α*$^{+f/+f}$ mutant animals breathing hypoxia have a higher level of plasma EPO than WT littermates at this early time point. Plasma EPO levels in the mutants do not change significantly with skin exposure to normoxia (Figure S2C). Thus the adaptation to skin oxygenation seen in WT animals in Figure 5B is eliminated when *HIF-1α* is absent from the epidermis. This indicates that HIF response in the skin in hypoxic adaptation is at least bipartite, with an early role in modulating EPO response (as shown in Figure S2C) and a later role in its maintenance (as shown in Figure 1C). This is a further demonstration of a central role for the epidermal HIF response in regulating adaptation to environmental oxygen levels.

DISCUSSION

Although the skin is essential for adaptation to environmental oxygenation in some other vertebrates, notably amphibians, no such role has been proposed before for mammals. Clearly, the physiologic function of mammalian skin differs in some respects from that of other vertebrates. It is also certain that an organisms' surface to volume ratio, its dermal vasculature, as well as its metabolic rate will influence how vascular conductance affects the hypoxic response that we demonstrate here. All of these will need to be taken into account in considering the relevance of these observations to other mammals. In addition, it should be noted that the differential oxygenation experiments were necessarily carried out under anesthesia, and thus some level of anesthesia-related change in vascular responsiveness could play a role in what we observed; further experimentation along these lines will be needed to establish how anesthetic agents might affect these results.

We show here that acute hypoxia reduces blood flow to the skin and that this in turn correlates with decreased renal EPO production; a longer term hypoxic challenge requires a HIF response in the skin to potentiate EPO production. These apparently disparate results both demonstrate an essential role for the skin in hypoxic EPO synthesis. Although this leads to the possibility of numerous complex models of response, a simple model to explain this disparity would be a dual role for the dermal hypoxic response in modulating circulation and EPO production (shown in Figure 6). Initially, hypoxic vasoconstriction acts in a rapid manner in the skin to increase flow to the kidney and modulate effects of decreases in blood pO_2. Regulation of

(E) The shift in blood flow corresponds to increased EF5 binding/hypoxia in the kidney and liver of *K14cre-VHL*$^{+f/+f}$ mice (WT, n = 8; *K14cre-VHL*$^{+f/+f}$, n = 4).
(F) Nitric oxide metabolites are increased in *K14cre-VHL*$^{+f/+f}$ plasma, demonstrating increased NO production (WT, n = 33; *K14cre-VHL*$^{+f/+f}$, n = 8). All graphs represent mean ± SEM.

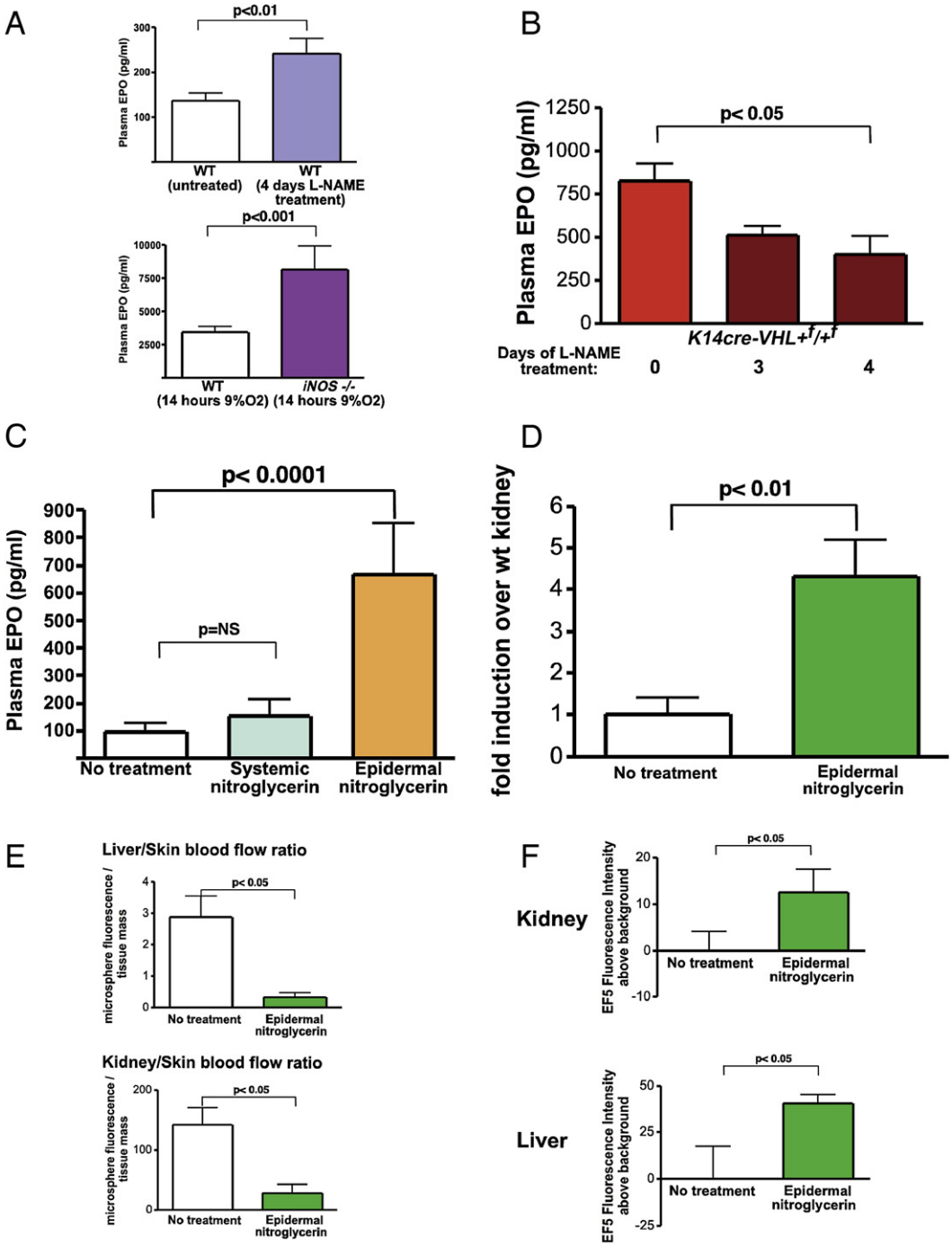

Figure 4. Nitric Oxide Production in the Skin Mediates Shift in Blood Flow and Renal EPO Expression in *K14cre-VHL*$^{+f/+f}$ Mice

(A) Systemic inhibition of NO synthesis by L-NAME increases plasma EPO in WT mice (WT, n = 36; WT + L-NAME, n = 12). Global *iNOS* knockout mice show significantly elevated EPO plasma levels following hypoxia relative to WT mice (WT, n = 25; *iNOS*$^{-/-}$, n = 7).

(B) NO synthesis inhibition by treatment with L-NAME restores plasma EPO levels to levels similar to L-NAME-treated WT mice, when administered to *K14cre-VHL*$^{+f/+f}$ mice (*K14cre-VHL*$^{+f/+f}$, n = 23; *K14cre-VHL*$^{+f/+f}$ + L-NAME 3 days, n = 3; *K14cre-VHL*$^{+f/+f}$ + L-NAME 4 days, n = 4).

(C) NO donor (nitroglycerin) applied to the skin of WT mice increases plasma EPO levels; similar systemic doses of NO donor do not significantly affect plasma EPO levels (control, n = 20; systemic nitroglycerin, n = 3; epidermal nitroglycerin, n = 9).

(D) Epidermal NO donor administration induces renal EPO mRNA expression (control, n = 9; epidermal nitroglycerin, n = 3).

(E) Epidermal nitroglycerin shifts blood flow (control, n = 11; epidermal nitroglycerin, n = 6) toward the skin and increases renal and hepatic hypoxia (F), in a manner similar to that seen in *K14cre-VHL*$^{+f/+f}$ mice (control, n = 8; epidermal nitroglycerin, n = 5). All graphs represent mean ± SEM.

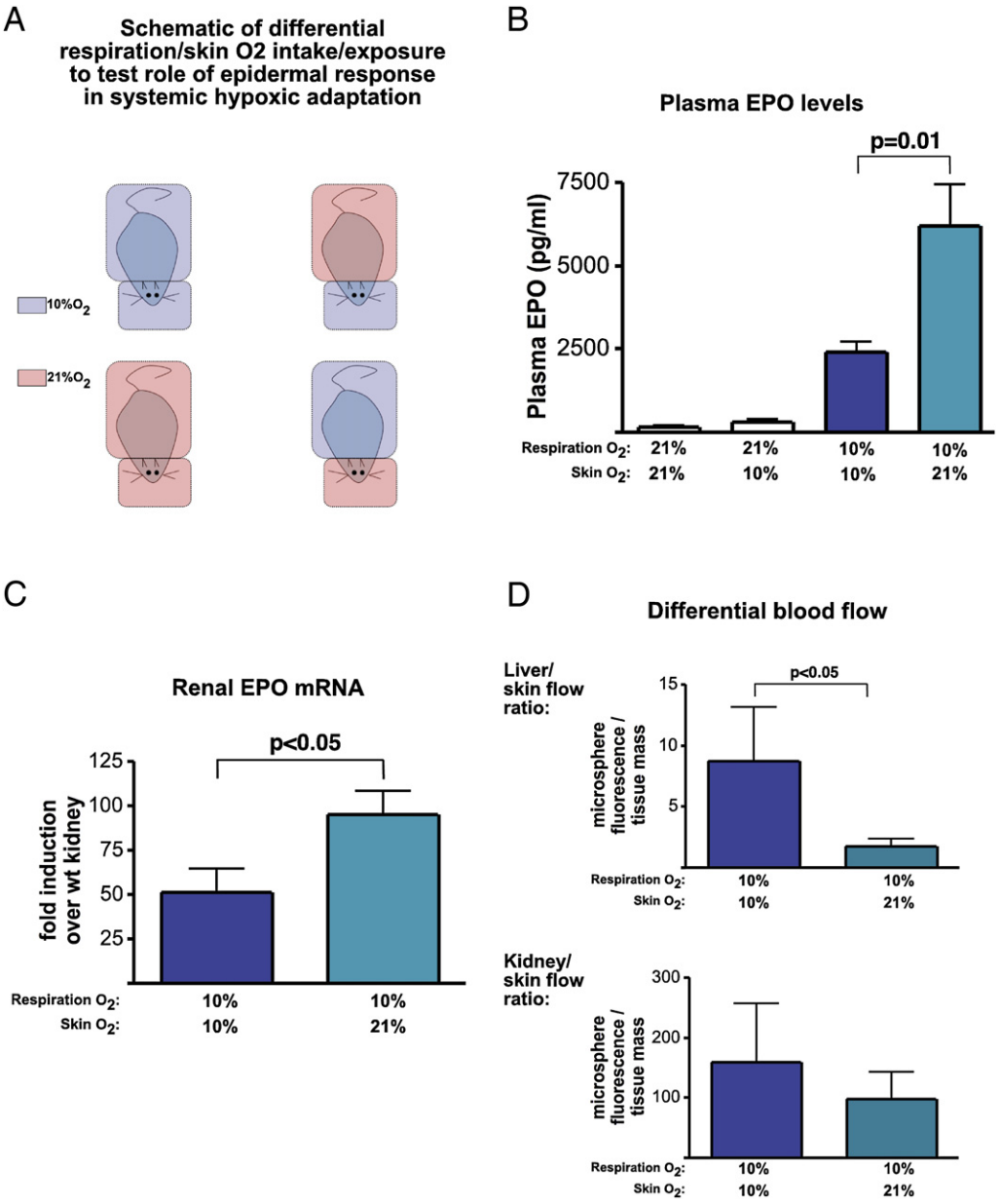

Figure 5. Skin Hypoxia Directly Affects Overall Systemic Hypoxic Response in Wild-Type Mice Exposed to Acute Hypoxia

(A) Special chambers were constructed to isolate inhaled oxygen concentration from the oxygen concentration exposed to the skin.

(B) In mice breathing 21% O_2, skin hypoxia was not enough to induce increased EPO synthesis. However, in mice breathing 10% O_2, plasma EPO levels were significantly elevated when skin was normoxic (respired 21% O_2, skin 21% O_2, n = 2; respired 21% O_2, skin 10% O_2, n = 3; respired 10% O_2, skin 10% O_2, n = 8; respired 10% O_2, skin 21% O_2, n = 9); mice were exposed to differential gases for 5 hr.

(C) Renal EPO expression was higher in mice breathing 10% O_2 while exposed to 21% O_2 (respired 10% O_2, skin 10% O_2, n = 6; respired 10% O_2, skin 21% O_2, n = 7); mice were exposed to differential gases for 5 hr.

(D) Blood flow is shifted from the skin to the kidney and liver when the skin is hypoxic for 1 hr. This shift is absent when the skin is exposed to normoxia (respired 10% O_2, skin 10% O_2, n = 7; respired 10% O_2, skin 21% O_2, n = 8). All graphs represent mean ± SEM.

potassium channel function and other HIF-dependent mechanisms of vasoconstriction (such as regulation of the HIF target gene endothelin-1) act to control vascular flow at this stage (Whitman et al., 2007). Transcriptional response through HIF in a more chronic hypoxic state would subsequently counteract this acute dermal vasoconstriction and over a period of hours gradually lessen blood flow to the kidney, acting to increase EPO production. This model argues for a dynamic role for both HIF and skin response to hypoxia in regulating internal blood flow during hypoxic response.

Hypoxia-induced pulmonary vasoconstriction (HPV) is essential to maintain blood flow to aerated sections of the lung following

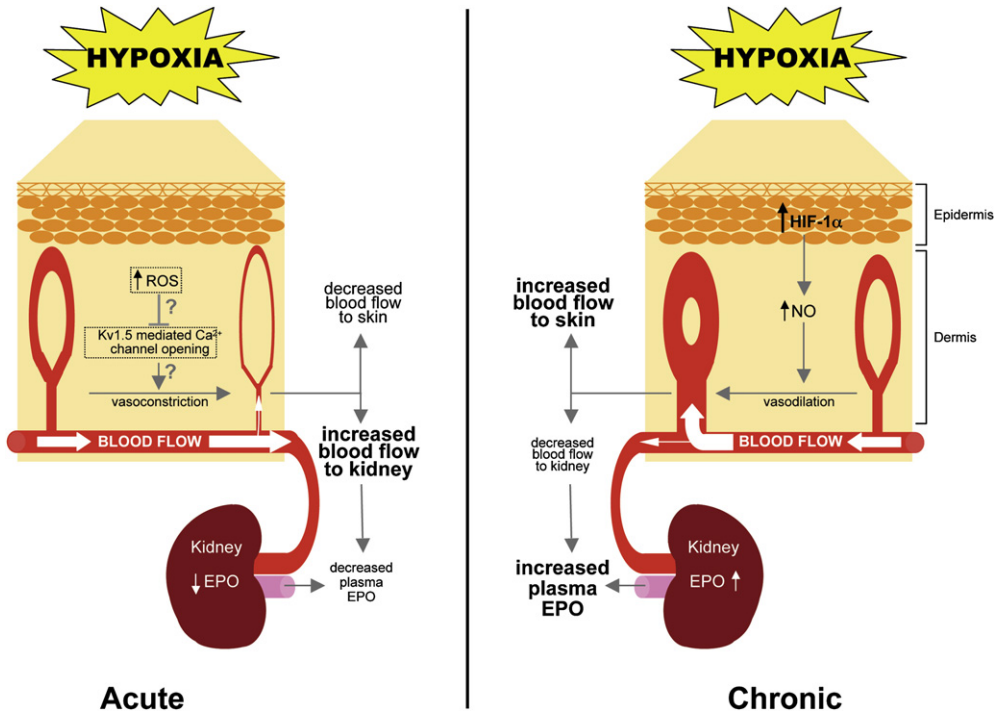

Figure 6. Acute and Chronic Adaptation to Hypoxia Is Influenced by Dermal Response

Hypoxia may act through mechanisms similar to those found in the lung to induce pulmonary vasoconstrictions under acute hypoxic stress; a more chronic stress allows a HIF-induced modulation of response that also affects blood flow and EPO expression.

injury or blockage of other regions. However, in generalized hypoxia, and in states of chronic and pervasive lung damage, it can lead to pulmonary edema and death (Weir et al., 2005). Although a number of mechanisms to explain HPV have been proposed, there is still considerable controversy about the nature of the oxygen-sensing mechanisms involved, as well as the various components required for the response (Weir et al., 2005). We have demonstrated the presence of one key modulator of HPV, the potassium channel Kv1.5, in dermal vasculature. Interestingly, Kv1.5 is also a HIF transcriptional target (Moudgil et al., 2006), as is a key modulator of voltage-gated potassium channels, the peptide endothelin-1 (Whitman et al., 2007). Further work on smooth muscle-specific knockouts of HIF factors may prove useful in delineating how the hypoxic response in the vasculature of the skin impacts the physiological response we have described.

Durand and Martineaud first showed in 1969 that humans demonstrate an immediate and persistent cutaneous vasoconstriction when exposed to high altitudes (Durand et al., 1969). Weil and colleagues subsequently found that although high altitudes resulted in cutaneous vasoconstriction in human subjects, similar levels of hypoxia inhaled at normal altitudes through breathing tubes did not alter dermal vascular tension (Weil et al., 1969). These data correspond to our findings in mice following acute exposure and indicate that similar mechanisms for hypoxia-induced skin vasoconstriction exist in humans. Further study will need to be done to show whether EPO levels are similarly regulated by dermal vascular flow in human subjects.

We demonstrate here a novel pharmacological mechanism for induction of EPO expression, application of a nitric oxide donor to the skin. We have also found that a number of factors that increase skin blood flow increase EPO expression in mice; one such factor is the compound allyl isothiocyanate (mustard oil) (Figure S1B). It is interesting to note that mustard oil massage of newborns is a widespread folk tradition among millions of people in Asia (Fikree et al., 2005; Mullany et al., 2005). It is intriguing to speculate that this massage could in part also influence neonatal erythropoietin synthesis.

In summary, mice regulate EPO response, and by extension systemic response to hypoxia, through a mechanism that is dependent on the skin and is correlated with changes in cutaneous blood flow. Thus, an intriguing role for the skin as an independent oxygen sensor and as a regulator of systemic response to environmental hypoxia is evident.

EXPERIMENTAL PROCEDURES

Mouse Breeding

The K14 cre mouse line (Haase et al., 2001; Jonkers et al., 2001) was obtained from A. Berns (Netherlands Cancer Institute). Intercrosses were generated by standard genetic techniques. K14 Tamoxifen-inducible cre mice (Vasioukhin et al., 1999) were a kind gift of E. Fuchs (Rockefeller). Control mice, when used for comparison to mutants, were always littermates of the mutant mice containing the same floxed alleles but *lacking* the *K14cre* transgene; they are referred to as "WT" for brevity.

Nitroimidazole Staining and Detection

The nitroimidazole EF5 and the anti-EF5 antibodies were provided by C. Koch (University of Pennsylvania). EF5 fluorescence for each section was calculated by subtracting the competed antibody intensity from primary antibody intensity.

Immunohistochemistry
Rabbit polyclonal *HIF-1α* antibody, a gift of R. Abraham (Burnham Institute), was used at 1:100 overnight. Anti-potassium channel Kv1.5 antibody (Chemicon) was used at 1:100.

Gene Deletion Efficiency
Deletion efficiency was determined by quantitative real-time PCR on genomic DNA isolated from the adult mouse epidermis.

EPO ELISA
EPO protein levels in blood plasma were determined with the Quantikine Mouse EPO ELISA kit (R&D Systems).

mRNA Expression
RNA was harvested from flash-frozen tissues using the Trizol reagent and protocol from Invitrogen. Primers were taken from Tam et al. (2006).

Blood Hematocrit and Volume
Hematocrit was measured by retro-orbital bleed into heparinized hematocrit tubes. Plasma volume was calculated by injecting 50 μl of 10 mg/ml Evans Blue dye i.v. into the tail vein and measurement of dilution.

Blood Oxygen Saturation
Measurements were made by pulse oximetery, using the MouseOx by Starr Life Sciences Corp.

Blood Pressure
Tail cuff measurements were made on the Kent Scientific XBP1000.

Blood Flow Analysis
Red fluorescent 15 μm FluoSpheres (Molecular Probes) were used. Fifty microliters of the microsphere mixture (50,000 spheres) was injected through the body wall into the left atrium of live mice immediately after experimental treatment; ratios of tissue microsphere fluorescence within mice were used to control for variations in microsphere injection efficiency. For skin harvest, the back of each mouse was shaved, and a 2 cm × 2 cm section of dorsal skin was taken.

Griess Assay
The measurement of plasma nitric oxide species was carried out according to Miranda et al. (2001).

L-NAME
L-NAME (Sigma) was administered in drinking water at 0.5 g/l for 3 or 4 days.

Nitroglycerin Experiments
3.5 cm^2 nitroglycerin patches (Hercon Laboratories) releasing 0.1 mg/hr for 7 hr were used for skin administration. To test the same dose orally we administered 0.7 mg of slow release Nitroglycerin (Ethex Corp.) by gavage; doses were calculated to approximate similar release rates. Blood was taken at the end of 7 hr by retro-orbital bleed or cardiac puncture.

Mustard Oil
Mustard oil (allyl isothiocyanate; Sigma) was diluted 1:10 in mineral oil. The dorsal skin of nude mice or shaved C57BL mice was painted with 10% mustard oil (MO) or mineral oil. Mice were painted five times over 7 hr.

Inhaled versus Skin Hypoxia Chambers
Individual mouse chambers were constructed from 50 ml Falcon tubes and plastic jars. Latex gaskets separated the body chamber from the head chamber. Mice were anesthetized with Ketamine/Xylazine during the experiment. Head and body chambers were perfused alternatively with 10% oxygen or 21% oxygen with significant positive pressure. Exhaled air was captured by a nose cone for metabolic measurements.

Statistics
All statistical analysis was performed using Prism (GraphPad Software). All error bars = SEM. All t tests are two-tailed unpaired t tests.

SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cgi/content/full/133/2/223/DC1/.

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